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Variability in tumor necrosis factor-α, nitric oxide, and xanthine oxidase responses to endotoxin challenge in heifers: Effect of estrous cycle stage☆

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Abstract

The severity of host response to some disease agents differs between sexes and this dimorphism has been attributed to the immunomodulating effects of steroid hormones. Our objective was to determine in heifers whether the phase of estrous cycle affected immune response mediators after endotoxin challenge (LPS, 2.5 μg/kg BW, i.v.). Sixteen beef heifers (426 ± 9 kg) were reproductively synchronized with the two-injection protocol of dinoprost tromethamine (Lutalyse®, Pfizer) to establish diestrus and estrus stages of the estrous cycle. Heifers were challenged with LPS on day 3 (E, estrus; $n = 8$) or day 10 (D, diestrus, $n = 8$) after the last i.m. injection of Lutalyse®. In all heifers, plasma concentrations of tumor necrosis factor-α (TNF-α) peaked 2 h after LPS treatment ($P < 0.01$) and returned to basal level by 7 h. However, the integrated TNF-α response (area under the time × concentration curve, AUC) was greater in E than in D ($P < 0.05$). Plasma concentrations of nitrate + nitrite (NO_x, an estimate of NO production) increased ($P < 0.01$) in all heifers at 7 and 24 h after LPS; plasma NO_x AUC after LPS was greater in E than D ($P < 0.01$). Plasma xanthine oxidase activity (XO, a mediator of superoxide production) responses were also greater in E than D ($P < 0.05$). A companion LPS challenge study in steers validated that the protocol for and use of Lutalyse® did not affect any of the immune parameters studied in heifers in response to LPS. Results indicate that the underlying physiological attributes of the estrus and diestrus phases of the estrous cycle constitute a major source of variability in the magnitude of proinflammatory response to bacterial toxins like LPS. Published by Elsevier Inc.

Keywords: Cattle; Estrous cycle; Endotoxin; Nitric oxide; Tumor necrosis factor-α

1. Introduction

Cytokine responses to provocative stress challenges modeled by endotoxin (LPS) administration have received great attention as biomarkers for and mediators of both homeostatic and pathophysiological processes *in vivo*. Several lines of evidence suggest that the outcome of some infections may be aligned with, and perhaps may be predicted by, plasma levels of cytokines during disease, particularly tumor necrosis factor-α (TNF-α; [1,2]). Transient bursts of TNF-α production are essential for initiating critical mediator response cascades of other cytokines, arachidonic/eicosapentanoic acid compounds, acute phase response proteins (APP), nitric oxide (NO), adrenomedullin [3,4], and activity of xanthine oxidoreductase (XO [5]), a major source of free

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radical superoxide. Superoxide has been shown to condense with NO *in vivo* to form peroxynitrite ($ONOO^-$) that may alter regulatory functions of targeted proteins by nitration or nitrosylation [6].

The severity of host response in some diseases differs between sexes and this dimorphism has been attributed to the immunomodulating effects of estrogens, androgens and progestins (for reviews see [7,8]). In general, estrogens enhance antibody production in human and animal models *in vivo* [9,10]. Peripheral blood lymphocytes isolated from cattle treated with an estradiol–progesterone metabolic repartitioning implant (Synovex-S®, 200 mg progesterone + 20 mg 17β-estradiol, Wyeth Pharmaceuticals-Fort Dodge Animal Health, Fort Dodge, IA), demonstrated enhanced antigen specific blastogenesis compared to cells from nonimplanted steers [11]. The mechanism of immunomodulating effects of estrogens during infection [12] or chronic inflammation [8] is very complex and involves a well coordinated temporally defined pattern of many pro- and anti-inflammatory cytokines [7,8], in addition to NO production via stimulation of NO synthases [13,14]. Progesterone (P4), on the other hand, has been suggested to transmit immunosuppressive signals during pregnancy [7] by decreasing production of inflammatory cytokines, particularly TNF-α [15]. There are also data to indicate anti-inflammatory properties of P4 in rodents including reduced production of TNF-α [16] and NO [17] from LPS-activated macrophages. In females, puberty, pregnancy, menopause, and age have been shown to affect the immune response to a disease stress through the prevailing sex steroid milieu [7,12,18].

The purpose of this study was to investigate in heifers whether the phase of estrous cycle affected the plasma concentration changes of immune response mediators after LPS challenge. Changes in the principal inflammatory initiation cytokine TNF-α, in two mediators involved in protein nitration (XO and NO), and in major anti-inflammatory hormone, cortisol, were studied.

2. Materials and methods

2.1. Animals and experimental design

All experiments were performed in accordance with approval of the Animal Care and Use Committee at the USDA Agricultural Research Service (Beltsville, MD, USA, Protocol #01-021). In the main experiment, sixteen beef heifers (age 10–12 months; 426 ± 9 kg) were fed individually a forage-concentrate diet (15% CP) to appetite and were synchronized to a similar stage of the estrous cycle with two-injection protocol (1st on day 0 and 2nd on day 11) for $PGF_{2\alpha}$ analog dinoprost tromethamine (Lutalyse®, Pharmacia & Upjohn Company, Kalamazoo, MI). Heifers were challenged with LPS (2.5 μg/kg BW; *E. coli* 055:B5, Sigma, St. Louis, MO; i.v. bolus via jugular catheters) 3 days (E, estrus; $n = 8$) or 10 days (D, diestrus; $n = 8$) after the last i.m. injection of Lutalyse®. For each challenge, jugular blood samples were obtained at 0, 1, 2, 3, 4, 7, and 24 h relative to LPS injection. Blood plasma samples were stored at −20 °C until assayed. Plasma progesterone (P4) concentrations before LPS challenge (at 0 h) were 0.3 ± 0.1 and 4.2 ± 0.6 ng/mL in E and D, respectively.

An auxiliary experiment was carried out to determine whether the time interval (3 days vs. 10 days) between the last Lutalyse® injection and LPS challenge would affect the magnitude of immune response to LPS challenge. Nine crossbred steers (age 10–12 months; 529 ± 10 kg) were fed a forage-concentrate diet (15% CP) to appetite and assigned to three different treatments ($n = 3$ per treatment) regarding Lutalyse® injection: no injection, injection 3 days or 10 days before LPS challenge. Protocol for LPS challenge and blood collection was the same as for the main experiment with heifers.

2.2. Plasma TNF-α determination

Immunoreactive plasma TNF-α concentration was measured in duplicate samples by specific double antibody RIA as previously described [19] using antisera generated in our laboratory to recombinant bovine TNF-α (Ciba-Geigy, Basel, Switzerland). Samples were assayed in a single batch with an intra-assay coefficient of variation less than 8%.

2.3. Xanthine oxidase determination

Plasma XO activities were determined in duplicate using Amplex™ Red Xanthine/Xanthine Oxidase Assay Kit (A-22182, Molecular Probes, Eugene, OR) validated for bovine plasma. Before the assay, plasma samples were diluted 1:10 (v/v) in 0.1 M Tris–HCl, pH 7.5. Samples were assayed in a single batch with an intra-assay coefficient of variation less than 5%.

2.4. Plasma nitrate + nitrite determination

The stable end-products of the NO pathway and markers of NO production, $NO_2^- + NO_3^-$ (NO_x), were measured using the Griess reaction after enzymatic conversion of plasma NO_3^- to NO_2^- with

nitrate reductase from Aspergillus species as previously described [20]. The intra-assay coefficient of variation was 9.5%.

2.5. Plasma cortisol (C) and progesterone (P4) determination

Plasma C and P4 concentrations were determined in duplicates using, respectively, solid-phase Active® Cortisol and Active® Progesterone RIA kits (Beckman Coulter, Diagnostic System Laboratories, Inc., Webster, TX) validated for bovine plasma. Intra-assay and inter-assay coefficients of variation were less than 7%.

2.6. Blood glucose determination

Blood glucose (Glu) concentrations were determined using the Accu-Chek® Advantage® human self-monitoring system (Roche Diagnostics Corp., Indianapolis, IN) adapted and validated for bovine blood samples [21]. The intra-assay coefficient of variation was equal to 3.2%.

2.7. Statistical analysis

Response to LPS challenge for plasma glucose, TNF-α, NO_x, and C concentrations and for plasma XO activity was calculated as area under the time × concentration curve (AUC) with baseline subtracted (concentrations at 0 h). The AUC for TNF-α and C response was calculated over the 7 h period and for NO_x and XO response over the 24 h period after each LPS challenge. The AUC for Glu was calculated during hyperglycemic phase only, i.e., over the 3 h period after each LPS challenge. Changes in plasma concentrations after LPS challenges were analyzed by a repeated measure ANOVA using the PROC MIXED procedure of SAS [22] with estrous cycle phase (D, E) and time after LPS challenge as fixed effects. Time after LPS challenge was considered repeated on the same animal which was nested within the cycle phase. When significant effects were detected ($P < 0.05$), differences between means were further separated by the ESTIMATE option of SAS. Response data (AUC) for all plasma parameters were compared between E and D heifers using the GLM procedure of SAS. Data are presented as least squares means ± SEM.

3. Results

The effectiveness of the synchronizing regimen to establish cattle in either the diestrus or estrus stage of the reproductive cycle was confirmed by measurement of plasma concentrations of P4. Plasma P4 concentrations (before LPS challenge, 0 h) were more than 12-fold higher in animals designated as diestrus (10 days post final Lutalyse® injection) as compared to those in estrus (3 days post final Lutalyse® injection); 0.3 ± 0.1 ng/mL versus 4.2 ± 0.6 ng/mL ($P < 0.005$) in E and D, respectively.

Administration of LPS to heifers (main experiment) and steers (auxiliary experiment) resulted in transient characteristic signs of mild systemic proinflammatory response. Mean rectal temperature increased 1.07 ± 0.11 °C after LPS challenge ($P < 0.01$, base temperature at 0 h vs. peak temperature at 4 h). Labored breathing accompanied by increased salivation, nasal discharge and coughing as well as mild diarrhea and lethargy were observed within 30 min of LPS administration; these empirically observed clinical signs resolved within 6–7 h after LPS challenge. No significant differences in the magnitude of the clinical signs were observed between heifers in E and D phase.

Increases in blood Glu concentration at 1 and 2 h after LPS administration ($P < 0.01$) were observed in all heifers and were followed by prolonged hypoglycemia ($P < 0.05$ vs. concentrations at 0 h) that lasted through 7 h (Fig. 1). However, with concentrations of Glu higher in E than D at 2 h ($P < 0.01$) and 3 h ($P < 0.05$) after LPS challenge, calculated blood Glu response (AUC) during hyperglycemic period (1–3 h) was greater (0 < 0.05) in E than D (Table 1).

Plasma concentrations of immunoreactive TNF-α increased ($P < 0.01$) in all heifers after LPS challenge (Fig. 2, upper panel). Peak concentrations were attained

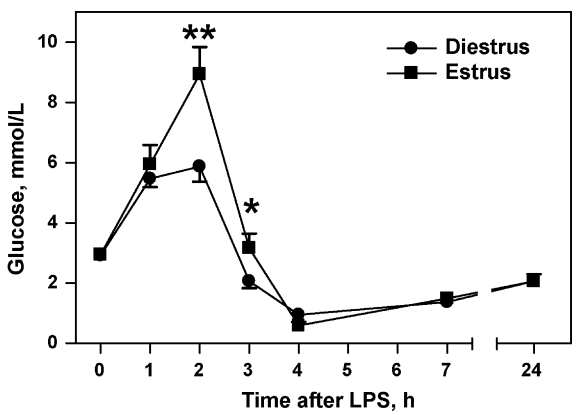

Fig. 1. Plasma glucose concentrations after LPS challenge (2.5 μg/kg BW, i.v.) during estrus (E) and diestrus (D) phase in heifers. Data represent least square means ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ between D and E at the same time.

Table 1
Integrated plasma responses (AUC[a]) to LPS challenge during estrus (E) and diestrus (D) phase in heifers.

Item[b]	Estrous phase		SEM[c]	$P <$
	D	E		
Glu (mmol/L × h)	5.09	9.09	1.12	0.05
TNF-α (ng/mL × h)	16.8	27.1	3.4	0.05
NO_x (μmol/L × h)	64.6	146.2	19.0	0.01
XO (mU/mL × h)	150.6	235.7	27.7	0.05
C (ng/mL × h)	159.2	129.5	11.4	0.08

[a] Response was calculated as area under the time × concentration curve (AUC) with baseline subtracted (period for AUC calculation after LPS challenge: 3 h for Glu, 7 h for TNF-α and C, 24 h for NO_x and XO).
[b] Glu: glucose; TNF-α: tumor necrosis factor-α; NO_x: $NO_2^- + NO_3^-$; XO: xanthine oxidase; C: cortisol.
[c] Common standard error of the mean from analysis of variance.

2 h after LPS and returned to the baseline by 7 h. However, mean TNF-α concentrations after LPS were higher in E than in D at 1, 2 h ($P < 0.01$), and 3 h ($P < 0.05$). Therefore, integrated plasma TNF-α response to LPS, calculated as area under the time × concentration curve (AUC), was higher ($P < 0.05$) in E than in D phase (Table 1).

After LPS challenge, plasma NO_x concentrations (Fig. 2, middle panel) increased, compared to 0 h, in all animals reaching peak at 7 h ($P < 0.05$) and values remained elevated at 24 h ($P < 0.05$). However, NO_x concentrations were lower in heifers in D than E at 7 h ($P < 0.01$) and 24 h ($P < 0.05$). Calculated total plasma NO_x response (AUC) to LPS challenge was lower ($P < 0.01$) in heifers in D than E (Table 1).

Plasma XO activities (Fig. 2, bottom panel) increased in all heifers starting 3 h after LPS challenge ($P < 0.05$ vs. time 0) but higher plasma activities were observed in E than D heifers at 7 h ($P < 0.05$) and 24 h ($P < 0.01$). Calculated plasma XO response (AUC) to LPS challenge was lower ($P < 0.05$) in D than E heifers (Table 1).

Plasma C concentrations (Fig. 3) were significantly ($P < 0.01$) increased in all heifers within 2 h of LPS exposure and remained higher than baseline concentration up to 24 h ($P < 0.01$). Plasma C concentrations 2 and 7 h after LPS challenge were higher ($P < 0.05$) in heifers in D than E and there was a trend ($P = 0.08$) toward a greater plasma C response (AUC) to LPS challenge in D than E (Table 1).

Administration of Lutalyse® 3 or 10 days before LPS challenge in steers (auxiliary experiment) did not affect Glu, TNF-α, NO_x, XO, and C response (AUC) to LPS challenge as compared to control steers not treated with Lutalyse® (Table 2).

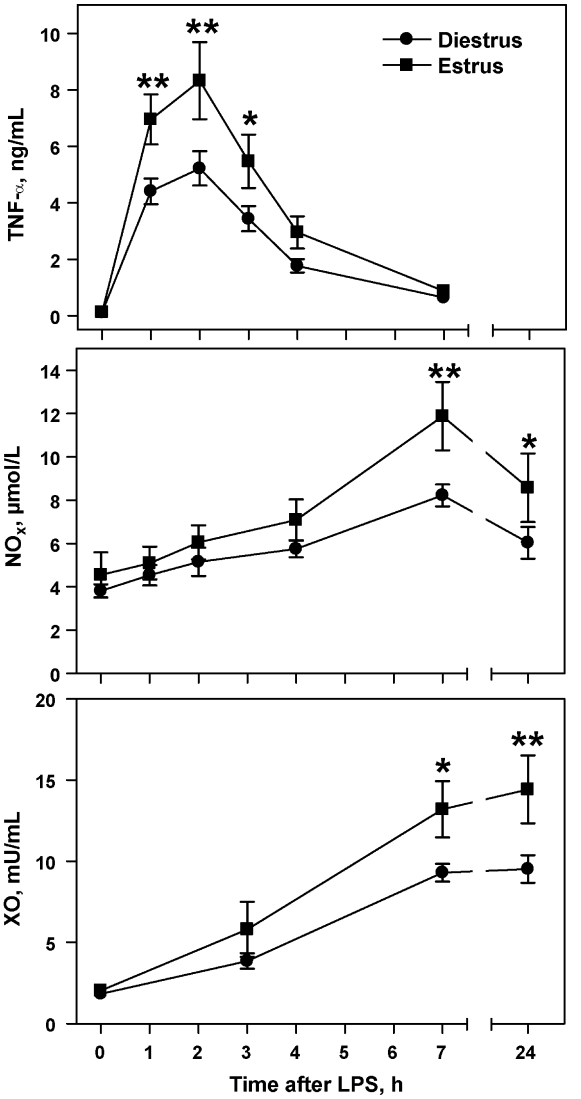

Fig. 2. Plasma concentrations of tumor necrosis factor-α (TNF-α, upper panel), nitrite + nitrate (NO_x, middle panel), and xanthine oxidase (XO, bottom panel) after LPS challenge (2.5 μg/kg BW, i.v.) during estrus (E) and diestrus (D) phase in heifers. Data represent least square means ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ between D and E at the same time.

4. Discussion

The intensity of response to infection has been shown to be different in many instances between males and females [7,8]. Previous studies from our laboratory [23] demonstrated that injection of testosterone to steers to achieve plasma concentrations similar to those measured in intact bulls resulted in an increase in the plasma concentrations of TNF-α and NO over those measured in steers. Two significant new findings of the present study on females elaborate upon and refine the observed

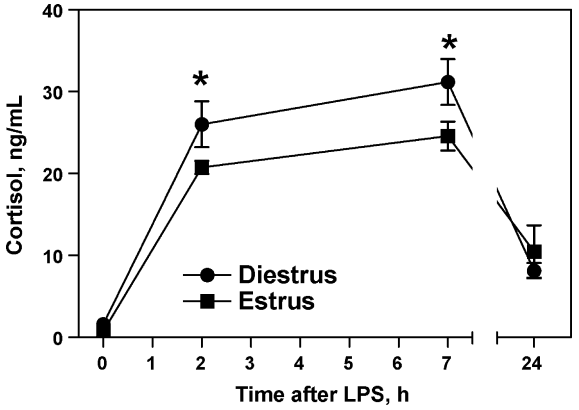

Fig. 3. Plasma cortisol (C) concentrations after LPS challenge (2.5 μg/kg BW, i.v.) during estrus (E) and diestrus (D) phase in heifers. Data represent least square means ± SEM ($n = 8$). $^{*}P < 0.05$ between D and E at the same time.

impacts of reproductive steroid hormone milieu on the magnitude of the proinflammatory response. First, the intensities of responses in proinflammatory mediators like plasma TNF-α and NO change throughout the estrous cycle. The implications of this impact the second point, i.e., interpretation of data surrounding the efficacy of intervention strategies intended to improve clinical outcome from the incurred proinflammatory response can be affected by the variability inherent in female subjects at different stages of the reproductive cycle. In the present study, the fact that the basic response in proinflammatory mediator concentrations changes were present as a function of the underlying endocrinological character of the two phases of the estrous cycle, implies that management strategies employing estrogen and/or progesterone-based pharmacological agents might affect the presentation of clinical signs of infection and proinflammatory response and in some cases be opportunistically exploited to improve the outcome.

Table 2
Integrated plasma responses (AUC[a]) to LPS challenge in control and Lutalyse® treated steers.

Item[b]	Treatment[c]			SEM[d]	$P =$
	CON	L03	L10		
Glu (mmol/L × h)	4.40	3.16	3.64	0.85	0.61
TNF-α (ng/mL × h)	6.78	5.80	6.17	0.71	0.63
NO_x (μmol/L × h)	102.1	111.7	99.3	13.3	0.79
XO (mU/mL × h)	187.7	221.0	195.6	29.5	0.72
C (ng/mL × h)	134.7	146.3	144.5	10.3	0.71

[a] Response was calculated as area under the time × concentration curve (AUC) with baseline subtracted (period for AUC calculation after LPS challenge: 3 h for Glu, 7 h for TNF-α and C, 24 h for NO_x and XO).

[b] Glu: glucose; TNF-α: tumor necrosis factor-α; NO_x: $NO_2^- + NO_3^-$; XO: xanthine oxidase; C: cortisol.

[c] Control (CON), no injection; Lutalyse® injection 3 (L03) or 10 (L10) days before LPS challenge.

[d] Common standard error of the mean from analysis of variance.

Data indicated that reproductive status in cows clearly affected the magnitude of the concentrations changes in plasma TNF-α response to immune challenge. Most interesting were the observations associated with the greater response in cows in E as compared with the D stage of the cycle. The more robust proinflammatory response of E animals may associate in parallel to previous observations where estrogenic growth promoters modulated a beneficial outcome to parasitic infection. Results of Heath et al. [24] indicate that the use of the estradiol-progesterone growth implant Synovex® (Wyeth Pharmaceuticals-Fort Dodge Animal Health, Fort Dodge, IA) decreases the severity of pathological response to infection with *Eimeria bovis* and improves the recovery of calves infected with coccidiosis as assessed by the lower fecal scores, improved mean weight retention and earlier return to full feed displayed by implanted animals. The beneficial effect of the estradiol component of Synovex® is consistent with other markers of improved immune function resulting from estrogen treatment [25,26] especially where selective and specific models and biomarkers are employed. Burton et al. [11] observed that the immunomodulatory effects of Synovex® can be rather specific as observed by a consistent and significant increase in antigen-specific blastogenic response in peripheral blood mononuclear cells (PBMC) obtained from implanted cattle immunized with ovalbumin in contrast to no significant effect on PBMC blastogenesis from either implanted of nonimplanted cattle receiving a standard mitogen (poke weed mitogen, concanavalin-A) blastogenesis challenge.

At the present time we can infer that the stronger immune response pattern observed at E stage might be beneficial towards the ability of the cycling female resisting the pathological consequences of infection and this may be a consequence of the prevailing estrogen milieu at this time. Supporting the positive health effect of the estradiol, data published by Kaushic et al. [27] indicated that infection of the reproductive tract with *Chlamydia trachomatis* was more severe in animals treated with P4 in contrast to the better outcomes present in animals treated with either estradiol alone or an estradiol-P4 combination. More recent data indicate that this estradiol-P4 combination specifically impacts aspects of the proinflammatory cascade down stream from the early-onset initiating proinflammatory cytokine release typified by TNF-α following an LPS challenge stimulus [28]. Specifically, Synovex® implantation of cattle

resulted in a significant downward modulation of NO and thromboxane generation after LPS. The significance of this down modulation might reside in lower generation of toxigenic reactive nitrogen intermediates derived from inappropriately large fluxes of NO in proximity to superoxide anion generation [29,30] as well as a more favorable balance in the prostacyclin/thromboxane ratio which, when out of balance, is causative to observed the pathological consequences of pulmonary hypertension and edema in the face of peripheral hypotension and multiple organ failure [31–33] commonly encountered in endotoxemia and septic crisis.

In the present experiment, lower response to LPS challenge observed both in general physiological parameters (e.g., hyperglycemia) and in primary immune mediators during diestrus also could be related to higher circulating concentrations of P4 as compared to estrus. Accumulated data suggest anti-inflammatory properties of P4 in rodents and humans, mainly through inhibition of production and release of a number of proinflammatory cytokines (for review see [8]) and inhibition of NO production [17]. This anti-inflammatory property of P4 is of special importance during pregnancy [15]. An interesting observation in the present study was that LPS challenge resulted in higher cortisol response during D than E phase. The role of cortisol as an anti-inflammatory hormone is well established [37] and modifications by gonadal hormones of hypothalamo-pituitary-adrenal axis response to immune challenge in rodents have been reported previously [38]. Progesterone, however, can modulate the response to LPS through at least two complementary mechanisms. These include the cross-binding of P4 to the anti-inflammatory glucocorticoid receptor and decreasing the expression of toll-like receptor-4 [39], the principle mediator of LPS recognition by several cell types [40]

The concept of hormonal balance in regard to immune function, however, cannot be underscored too strongly. A tremendous range of possible immune response changes can be attributed to estradiol or P4 especially in regards to where a break point can be established beyond which benefit gives way to frank perturbation. Such breakpoints are apparently reached under conditions where ethinyl estradiol treatment is sufficiently intense so as to increase hepatocyte mitochondrial superoxide anion generation and free radical tissue damage and mitochondria-driven apoptosis [34]. By comparison, a long-term treatment of low dose estradiol appears to have an opposite effect where an estrogen-associated reduction in apoptotic factors provides a significant measure of increased survival during congestive heart failure [35]. The critical feature here revolves around two facts. First, as reviewed by Ahmed et al. [25], subpopulations exist where modulation of the sex steroid milieu towards a more estrogen dominant milieu is associated with increased occurrence of aberrant consequences as exemplified by autoimmune dysfunction, diseases recognized as significantly more prevalent in females than males. Second, care need be taken in extrapolating generalizations of benefit or detriment estrogen modulation of immune function across species or even pharmacological composition where, as stated by Cave et al. [36], “unexpected outcomes” can lend uniqueness to a particular species.

In conclusion, this study underscores the need to consider the estrous cycle phase in cow as a major source of variability in the magnitude of immune response to bacterial toxins like LPS. Increases in the circulating concentrations of the primary inflammatory cytokine TNF-α and two mediators (XO and NO) responsible for the production of reactive nitrogen and oxygen species and protein nitration were higher during estrus than diestrus. This difference in responses between cycle phases appears to be related to the endocrine environment, particularly P4 concentrations, as P4 and cortisol interact to shape the intensity of the proinflammatory response.

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